

SUPPLEMENTARY INFORMATION

A self-labeling protein based on the small ultra-red fluorescent protein, smURFP.

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1. Chemical Synthesis

1.1. General chemicals & reagents

Chemical synthesis was performed using 5(6)-Carboxyfluorescein (**FAM**, Millipore Sigma 21877), 2,2'-(ethylenedioxy)bis(ethylamine) (**PEG**, Millipore Sigma 385506), biliverdin hydrochloride (Frontier Scientific B655-9), dimethylformamide (DMF, Millipore Sigma 270547), pyridine (py, Millipore Sigma 270970), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Oakwood Chemicals 024810), hydroxybenzotriazole (HOBt, Oakwood Chemicals M02875), dimethyl sulfoxide- d_6 (Acros Organics AC321290100), dimethyl sulfoxide (DMSO, Millipore Sigma D8418), acetonitrile (ACN, Millipore Sigma 34851), and trifluoroacetic acid (TFA, Millipore Sigma 302031).

The following molecular biology reagents were used in this research; LB/agar (Millipore Sigma L2897), LB (Millipore Sigma L3522), ampicillin (Millipore Sigma A1593), arabinose (Millipore Sigma A91906), BPER (ThermoFisher, 78243), DNaseI (ThermoFisher EN0521), Trizma Base (Millipore Sigma T1503), NaCl (Millipore Sigma S7653), imidazole (Millipore Sigma RDD044), and phosphate buffered saline (PBS, ThermoFisher, 10010023).

Cell culture and fluorescence imaging reagents were HEK293A cells (ThermoFisher R70507), DMEM with no phenol red (ThermoFisher 11054020), fetal bovine serum (FBS, ThermoFisher 10438026), Penicillin, Streptomycin (Millipore Sigma P4333), GlutaMAX (ThermoFisher 35050061), Opti-MEM (ThermoFisher 31985070), and 10 mg/mL of Hoechst 33342 in water (ThermoFisher H3570).

1.2. High performance liquid chromatography & mass spectrometry

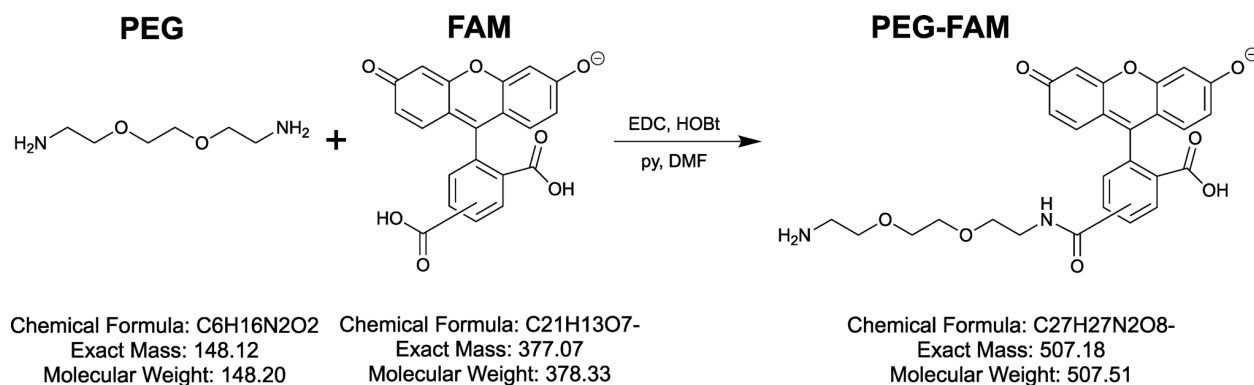
Analytical high performance liquid chromatography (HPLC) was performed using an Agilent 1100 Series HPLC coupled to a linear single quadrupole detector, an electrospray ionization source, and UV absorption detector (HPLC-MS, Agilent LC/MSD trap XCT). The reverse phase Luna C18(2) column (Phenomenex, 00G-4252-E0) had a 5 μm particle size, 100 Å pore size, 25 cm length, and 4.6 mm internal diameter. The flow rate was set to 1 mL/min with a 10-90% water (H_2O):acetonitrile (ACN) gradient + 0.05% trifluoroacetic acid (TFA) for 20 or 40 min runs. Absorbance was detected at 207-223, 278-282, 645-655, and 775-785 nm, and mass spectrometry was performed in positive ion mode.

Preparative HPLC was performed using an Agilent 1200 Series HPLC with a reverse phase Luna C18(2) column (Phenomenex, 00G-4253-P0-AX) with a 10 μm particle size, 100 Å pore size, 25 cm length, and 21.2 mm internal diameter. A 15 mL/min flow rate and a 20 minute, a 10-90% H_2O :ACN gradient + 0.05% TFA was used for purification. Fractions were collected, combined, and lyophilized. Typical isolated, lyophilate yields were 20-40% for each reaction.

1.3. Synthesis of BV-PEG-FAM

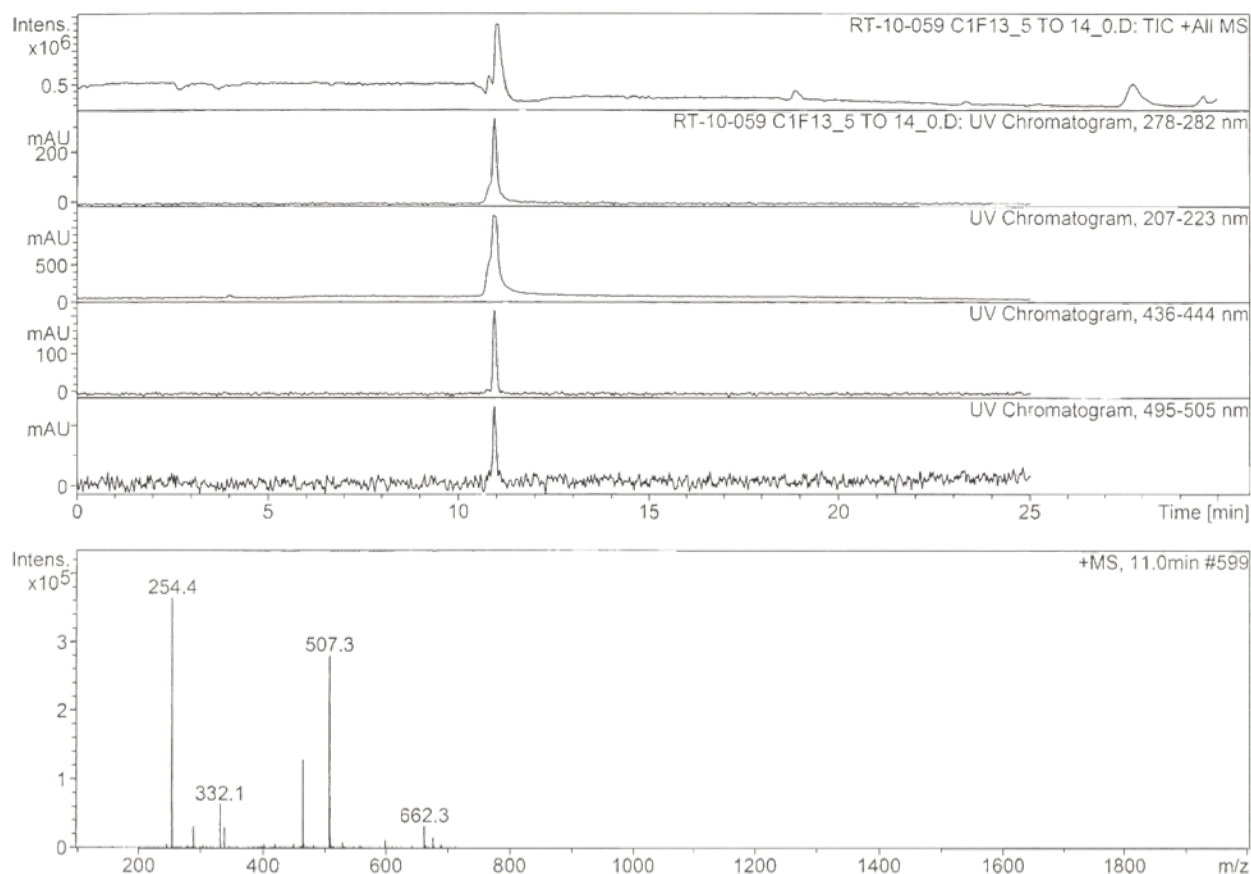
1.3.1. Synthesis of PEG-FAM

5(6)-Carboxyfluorescein (**FAM**, 230 mg) was added to a vial. 2,2'-(ethylenedioxy)bis(ethylamine) (**PEG**, 6.82 M, 90 mL), dimethylformamide (DMF, 700 mL), pyridine (py, 70 mL), hydroxybenzotriazole (HOBt, 3 mg), and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 300 mg) were sequentially added and sonicated for 60 minutes using the Branson 2510 sonicator (**Supporting Scheme 1**).



Supporting Scheme 1 Synthesis of PEG-FAM.

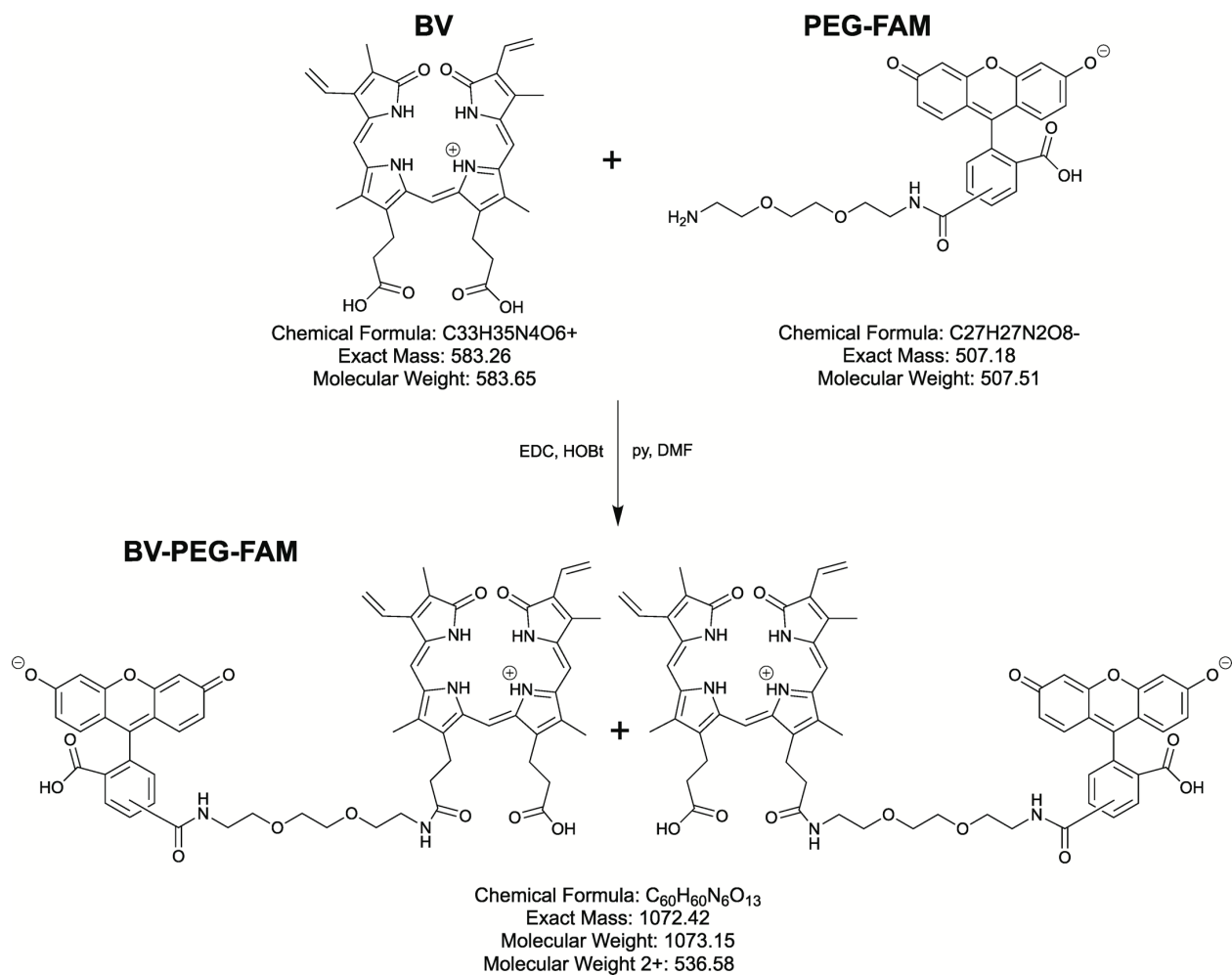
A preparative HPLC was performed as described in **Section 1.2** and retention times of 13.50-14.00 minutes were combined and lyophilized. An analytical HPLC – MS⁺ was performed as described in **Section 1.2** and shown in **Supporting Fig. 1**. UV-absorbance at 492 nm indicates **FAM** at a retention time of 11 minutes and confirmed by MS.



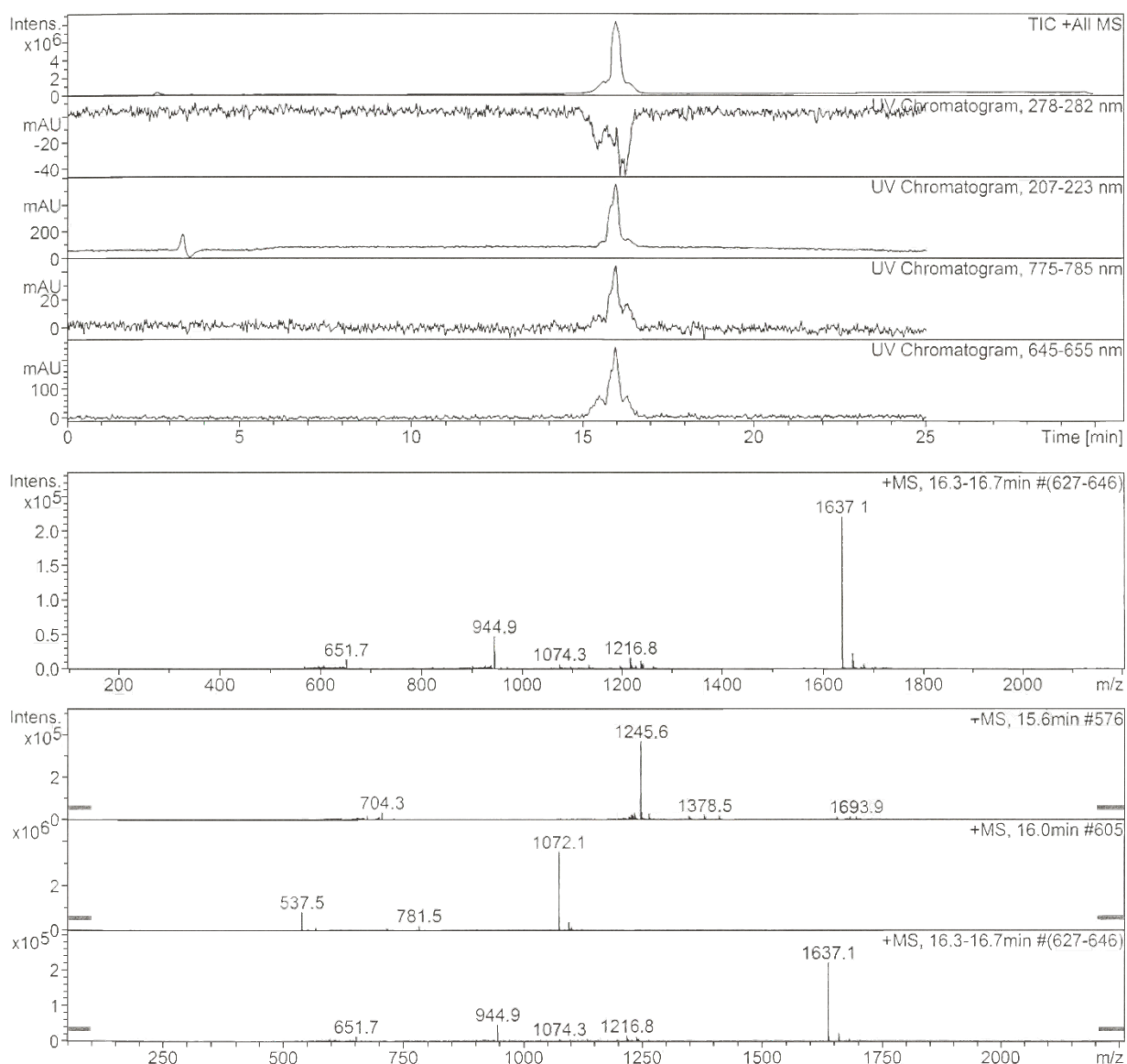
Supporting Fig. 1 Analytical HPLC – MS⁺ of **PEG-FAM**. Fractions taken from the preparative HPLC from 10.75-11.25 minute retention times contained **PEG-FAM** and were run on an HPLC using H₂O:ACN (a10-90%) + 0.05% TFA, 40 min gradient, 1 mL/min flow rate, and absorbance detected at 280, 215, 440, and 500 nm. The predicted molecular weight (M/Z) is 507.51 and the measured M/Z was 507.3.

1.3.2. Synthesis of BV-PEG-FAM

Biliverdin hydrochloride (BV, 5 mg), **PEG-FAM** (6.2 mg), HOBt (2 mg), DMF (500 mL), pyridine (py, 50 mL), and EDC (7 mg) were sequentially added to a vial followed by 120 minutes sonication (**Supporting Scheme 2**). A preparative HPLC and analytical HPLC – MS⁺ was performed as described in **Section 1.2**. **BV-PEG-FAM** was taken from retention time of 16.0 min on preparative HPLC and lyophilized. Analytical HPLC – MS⁺ is shown in **Supporting Fig 2**.



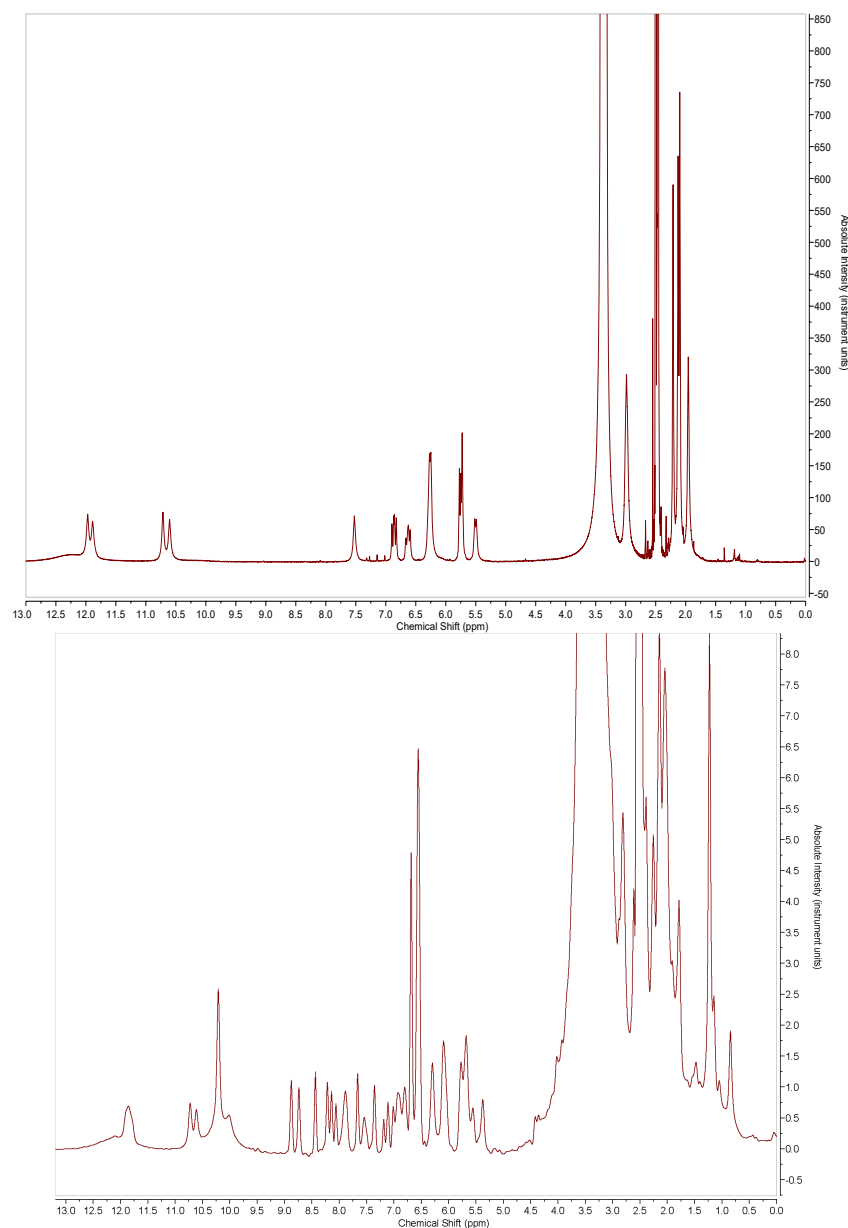
Supporting Scheme 2 Synthesis of **BV-PEG-FAM**.



Supporting Fig. 2 Analytical HPLC – MS⁺ of BV-PEG-FAM. Sample from retention time of 16.0 min on the preparative HPLC. The analytical HPLC was run with H₂O:ACN (a10-90%) + 0.05% TFA, 40 min gradient, 1 mL/min flow rate, and absorbance detected at 280, 215, 780, and 650 nm. The predicted molecular weight (M/Z) is 1,073.15 and an M/Z was found at 1072.1. The doubly charged peak (M²⁺) was found at 537.5.

1.3.3. Proton NMR of BV and BV-PEG-FAM

BV, starting material, and **BV-PEG-FAM** proton NMR were taken on Agilent Technologies 400 or 600 MHz ^1H -NMR at 20 °C. 50 μL of 5 mM BV and **BV-PEG-FAM** was lyophilized on a FreeZone 1 Liter Benchtop Freeze Dry System. The dry product was dissolved in 500 μL of deuterated dimethyl sulfoxide (DMSO-d_6) and transferred to a 5 mm NMR tube to acquire the proton NMR with TMS reference. The files were processed using MestReNova (12.0.4-22023). Peak selections, multiplet detections, and integrations were performed manually in MestReNova. **Supporting Fig. 3** shows NMR spectra.

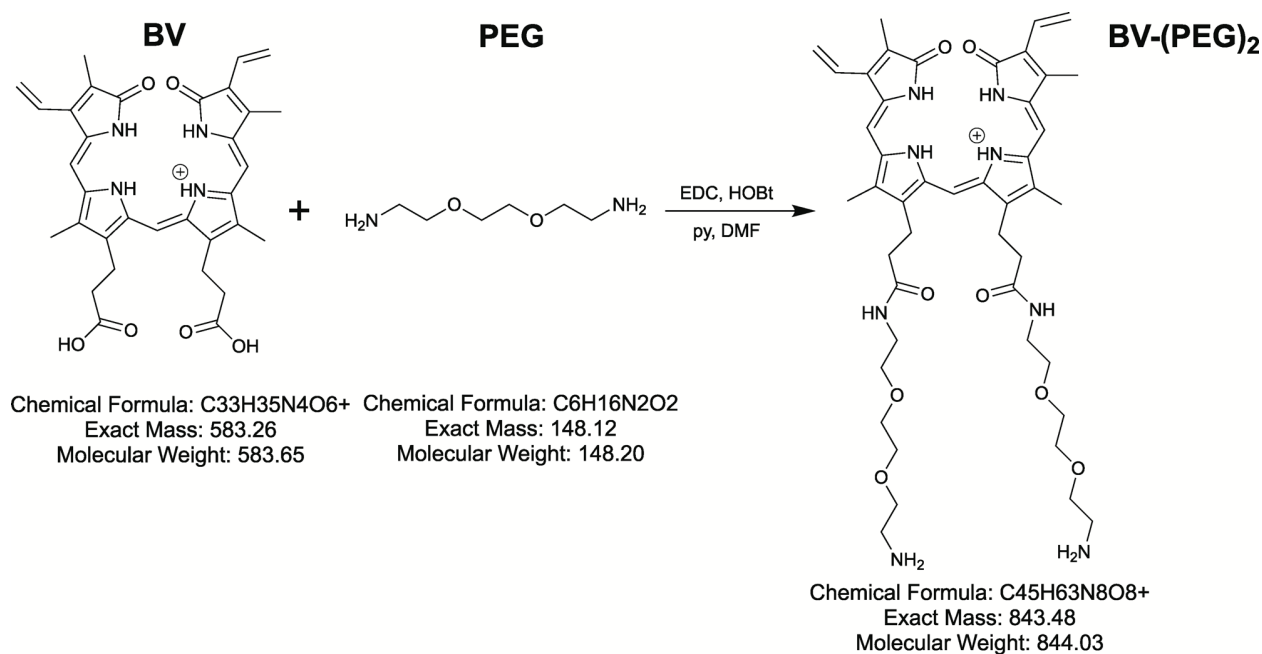


Supporting Fig. 3 ^1H NMR of BV taken at 400 MHz in DMSO- d_6 at 20 $^\circ\text{C}$ (**Top**) and BV-PEG-FAM taken at 600 MHz in DMSO- d_6 at 20 $^\circ\text{C}$ (**Bottom**). (**Top**) BV: δ 12.85-12.07 (m, 1H), 12.06-11.93 (s, 1H), 11.93-11.72 (s, 1H), 10.85-10.66 (s, 0.9H), 10.66-10.40 (s, 0.9H), 7.69-7.35 (s, 0.9H), 6.95-6.77 (dd, 1H), 6.71-6.55 (m, 0.9), 6.42-5.87 (d, 1H), 5.86-5.74 (d, 1H), 5.74-5.61 (s, 1H), 5.61-5.34 (d, 1H), 3.06-2.86 (s, 4H), 2.31-2.17 (s, 3H), 2.16-2.11 (s, 3H), 2.11-2.03 (s, 3H), and 2.02-1.67 (s, 3H). (**Bottom**) BV-PEG-FAM: δ 12.84-12.03 (s, 0.9H), 12.02-11.62 (m, 2H), 11.08-10.46 (d, J = 6.83 Hz, 1H), 8.01-7.76 (m, 1H), 7.76-7.61 (s, 0.6H), 7.60-7.44 (m, 0.5H), 7.43-7.26 (s, 0.5H), 6.96-6.85 (m, 1H), 6.85-6.63 (s, 3H), 6.63-6.47 (s, 5H), 6.40-6.21 (s, 1H), 6.21-5.88 (s, 2H), 5.85-5.73 (m, 1H), 5.73-5.63 (s, 1.6H), 5.63-5.49 (s, 0.7H), 2.32-2.21 (s, 4.7H), 2.21-2.10 (s, 7.6H), 2.09-1.95 (s, 9.7H), 1.86-1.72 (s, 4.3H), 1.34-1.10 (d, J = 4.83 Hz, 8H). The proton numbers are non-integers because **FAM** is a mixture of the 5-carboxyfluorescein and 6-carboxyfluorescein isomers.

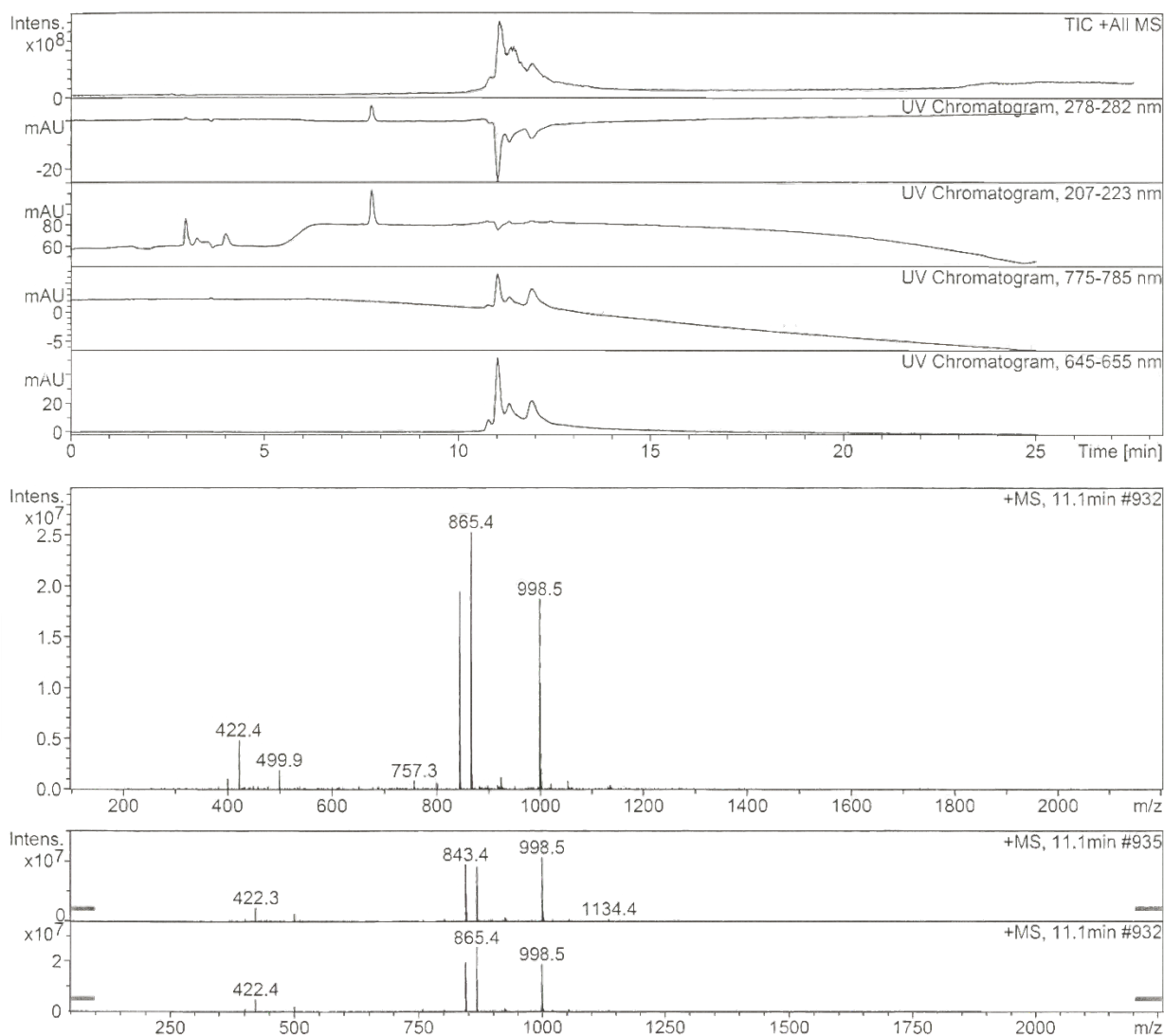
1.4. Synthesis of BV-(PEG-FAM)₂

1.4.1. Synthesis of BV-(PEG)₂

Biliverdin hydrochloride (24.3 mg), DMF (300 mL), **PEG** (8 mL), py (32 mL), HOBT (8 mg), and EDC (32 mg) were added sequentially to a vial followed by a 60 minute sonication using the Branson 2510 sonicator (**Supporting Scheme 3**). A preparative HPLC and analytical HPLC – MS⁺ was performed as described in **Section 1.2**. **BV-(PEG)₂** was taken from retention time of 11.0 min on preparative HPLC and lyophilized. Analytical HPLC – MS⁺ is shown in **Supporting Fig 4**.



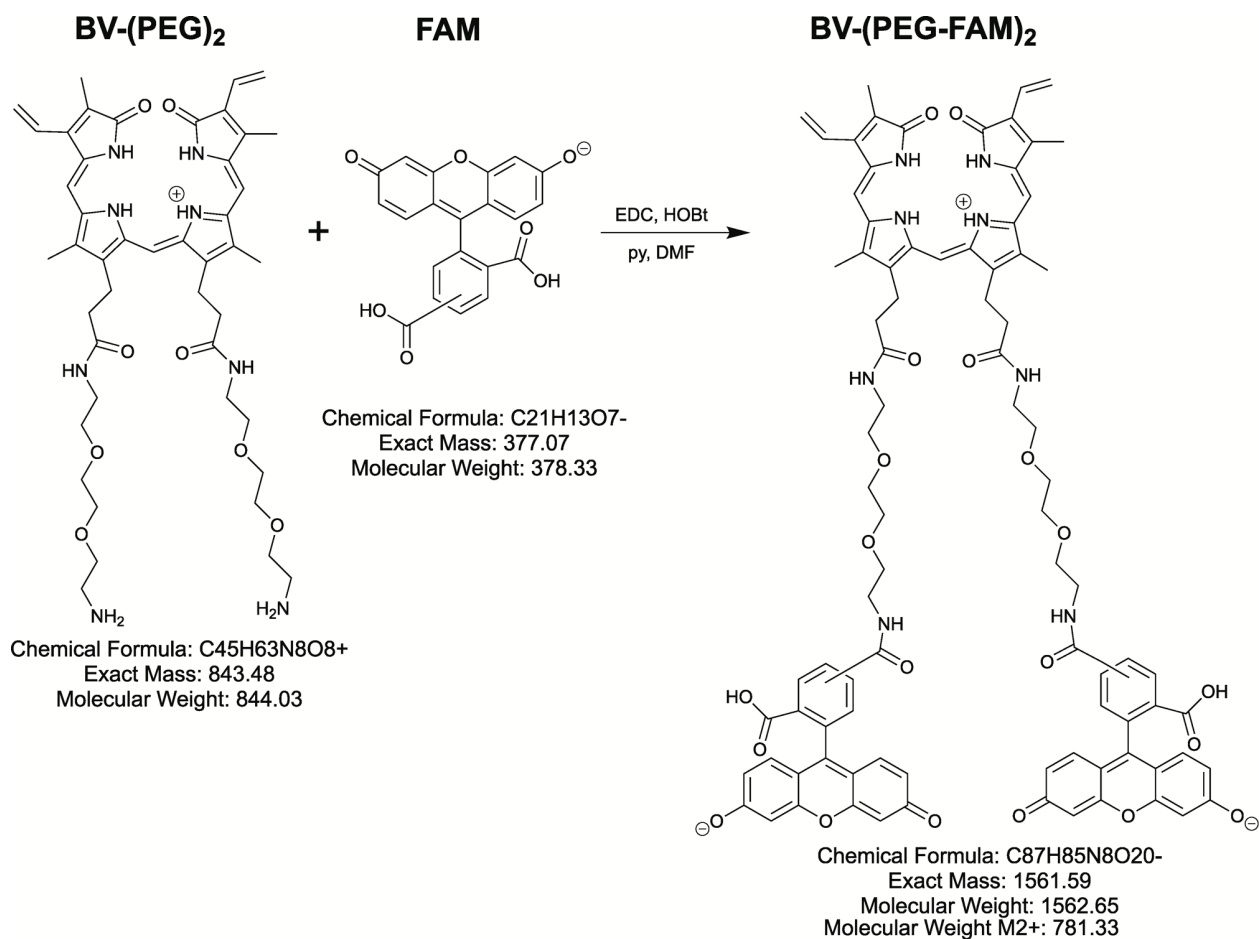
Supporting Scheme 3 Synthesis of **BV-(PEG)₂**.



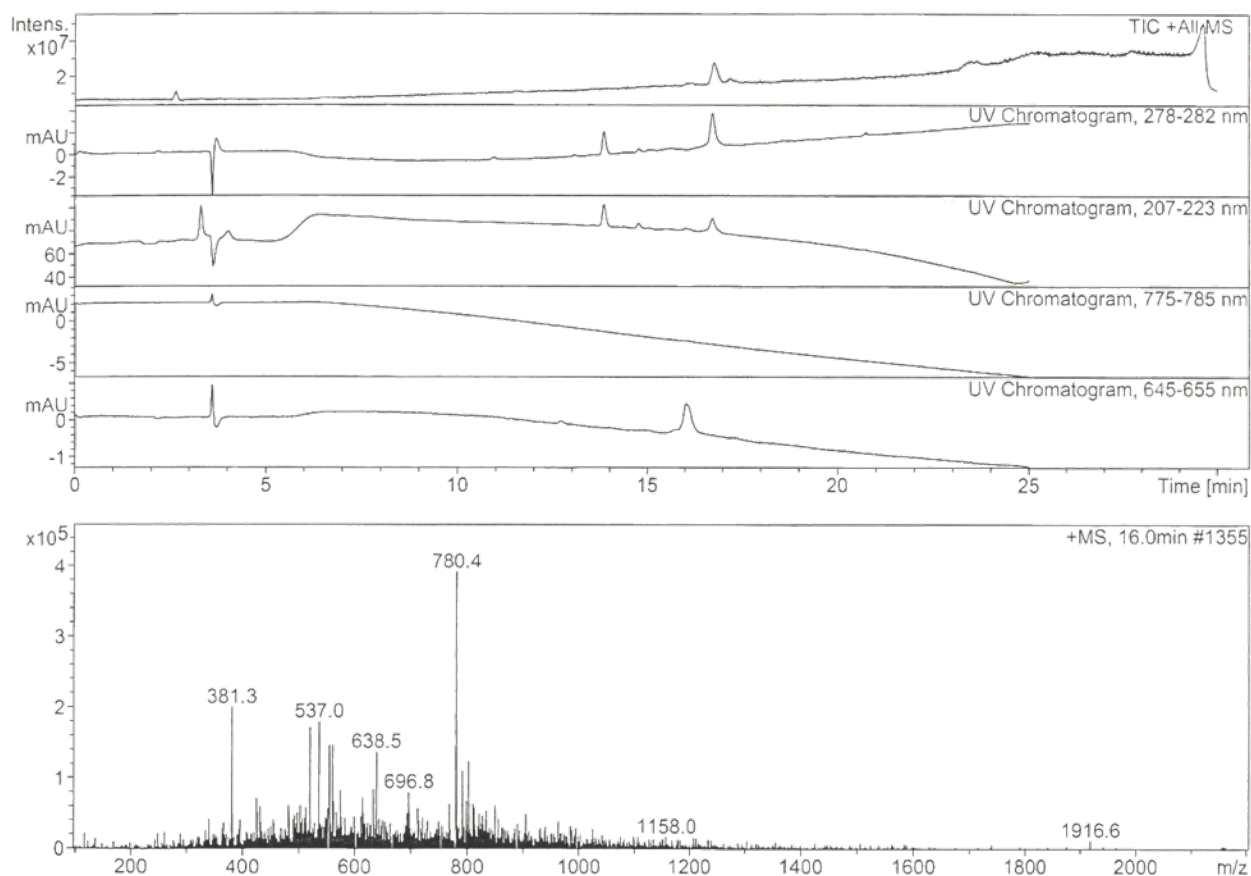
Supporting Fig. 4 Analytical HPLC – MS⁺ of BV-(PEG)₂. Preparative HPLC fractions were taken at retention time of 11.0 min. Analytical HPLC was run with H₂O:ACN (a10-90%) + 0.05% TFA, 20 min gradient, 1 mL/min flow rate, and absorbance detected at 280, 215, 650, and 780 nm. The predicted molecular weight (M/Z) is 844.03 and an M/Z was found at 843.4.

1.4.2. Synthesis of BV-(PEG-FAM)₂

BV-(PEG)₂ (3 mg), 5(6)-Carboxyfluorescein (**FAM**, 6 mg), DMF (200 mL), py (20 mL), HOBt (3 mg), and EDC (3 mg) were added sequentially to a vial and sonicated (**Supporting Scheme 4**). A preparative HPLC and analytical HPLC – MS⁺ was performed as described in **Section 1.2**. **BV-(PEG-FAM)₂** was taken from retention time of 16.0 min on preparative HPLC and lyophilized. Analytical HPLC – MS⁺ is shown in **Supporting Fig 5**.



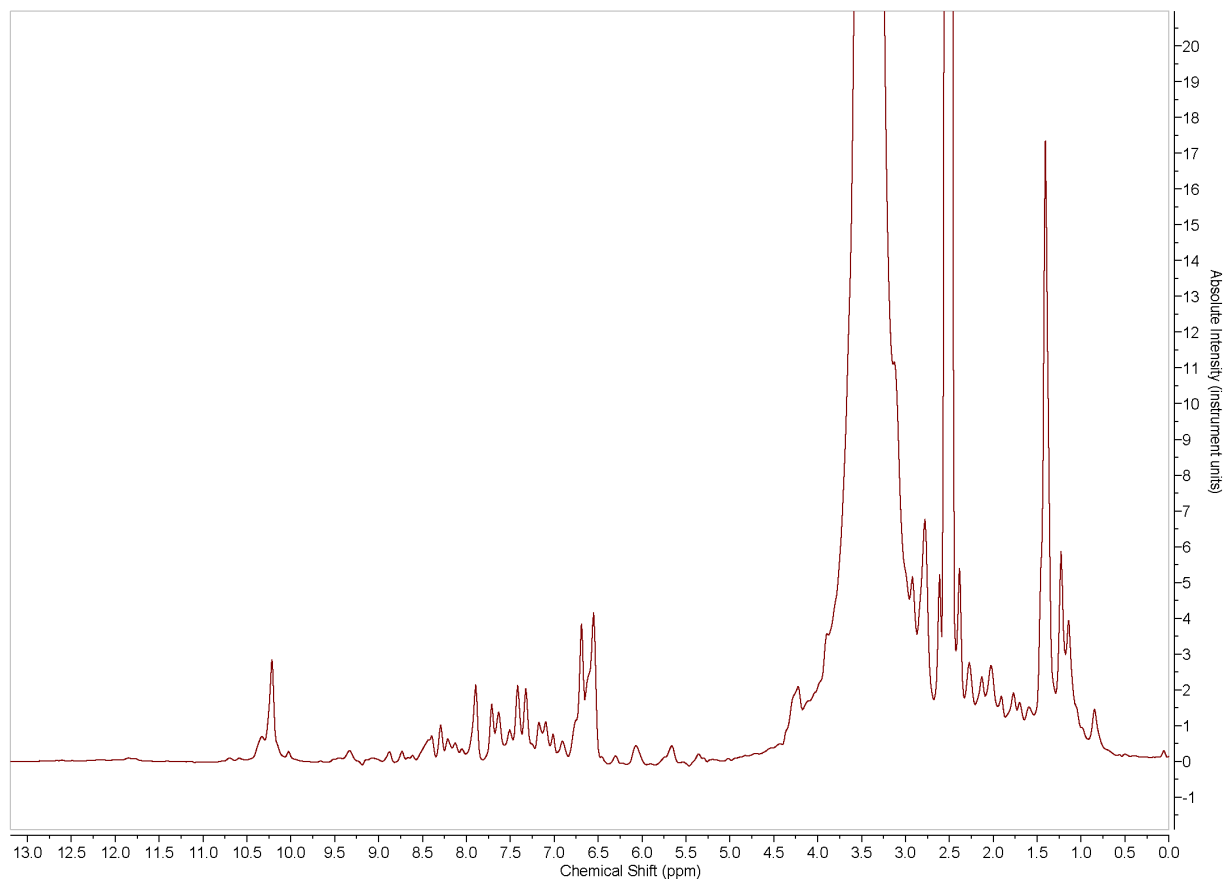
Supporting Scheme 4 Synthesis of **BV-(PEG-FAM)₂**.



Supporting Fig. 5 Analytical HPLC – MS⁺ of **BV-(PEG-FAM)₂**. Preparative HPLC fractions were collected at 16.0 min. The analytical HPLC was run with H₂O:ACN (a10-90%), 0.05% TFA, 40 min gradient, 1 mL/min flow rate, absorbance detected at 280, 215, 650, and 780 nm. The predicted molecular weight (M/Z) is 1562.65 and the doubly charged peak (M²⁺) was predicted as M/Z of 781.33 and the M/Z was found at 780.4.

1.4.3. Proton NMR of **BV-(PEG-FAM)₂**

BV-(PEG-FAM)₂ proton NMR was taken on Agilent Technologies 600 MHz ¹H-NMR at 20 °C. 20 µL of 5 mM BV and **BV-PEG-FAM** was lyophilized on a FreeZone 1 Liter Benchtop Freeze Dry System. The dry product was dissolved in 500 µL of deuterated dimethyl sulfoxide (DMSO-*d*₆) and transferred to a 5 mm NMR tube to acquire the proton NMR with TMS reference. The files were processed using MestReNova (v 12.0.4-22023). Peak selections, multiplet detections, and integrations were performed manually in MestReNova. **Supporting Fig. 6** shows the NMR spectrum.



Supporting Fig. 6 ^1H NMR of **BV-(PEG-FAM)₂** taken at 600 MHz in DMSO- d_6 at 20 °C. **BV-(PEG-FAM)₂**: δ 10.77-10.51 (m, 0.15) 8.00-7.81 (s, 1.6H), 7.80-7.56 (d, J = 4.75 Hz, 2H), 7.55-7.46 (s, 0.7H), 7.47-7.26 (d, J = 5.56 Hz, 2.9H), 6.98-6.73 (s, 1.4H), 6.72-6.65 (s, 4H), 6.65-6.49 (s, 4H), 6.15-5.95 (s, 0.3H), 5.72-5.95 (s, 0.3H), 2.33-2.21 (s, 3H), 2.20-2.10 (s, 2.3H), 2.09-1.96 (s, 3H), 1.95-1.86 (s, 1.5H), and 1.53-1.29 (s, 16H). The proton numbers are non-integers because **FAM** is a mixture of the 5-carboxyfluorescein and 6-carboxyfluorescein isomers.

2. BV-PEG-FAM covalent attachment to smURFP-tag *in vitro*

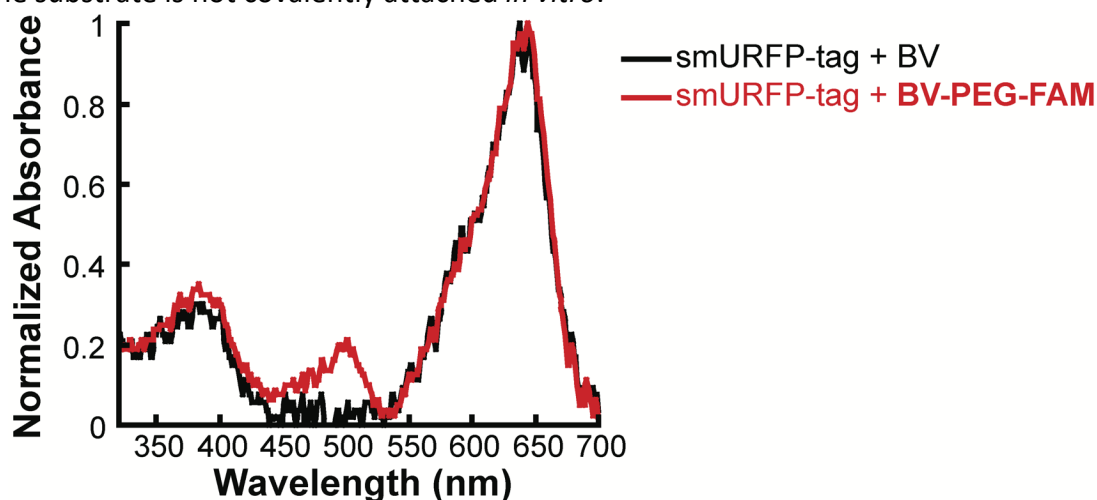
2.1. Purification of smURFP-tag without chromophore

smURFP-tag was purified from *E. coli* using pBAD smURFP RBS HO-1 (Addgene 80341) digested with MscI (NEB, R0534) and PmeI (NEB, R0560) to remove the C-terminus of the HO-1 gene¹. Fresh, chemically competent Top10 *E. coli* (ThermoFisher C404010) were transformed and plated on LB/agar plates containing 50 $\mu\text{g}/\text{ml}$ ampicillin and 0.02% arabinose to induce smURFP expression. Colonies were white and non-fluorescent, indicative of smURFP expression without BV chromophore. A single colony was added to 1 L of LB containing 50 $\mu\text{g}/\text{ml}$ ampicillin and 0.02% arabinose and grown for three days at 37 °C with shaking. *E. coli* were pelleted and lysed with BPER + DNaseI for 1 h at room temperature with shaking. Insoluble material was pelleted by centrifugation at 4 °C, and the supernatant was added to NiNTA agarose beads (G-

Biosciences 786-940) washed with 50 mM Tris, 300 mM NaCl, and 10 mM imidazole (pH = 7.5). smURFP was allowed to bind at room temperature with gravity flow. The bound smURFP was washed with 50 mM Tris, 300 mM NaCl, and 10 mM imidazole (pH = 7.5), eluted with 50 mM Tris, 300 mM NaCl, and 200 mM imidazole (pH = 8), and immediately buffered exchanged with PD-10 Desalting Columns (GE Healthcare, 17-0851-01) into phosphate buffered saline (PBS, pH 7.4). Protein concentration was determined by absorbance at 280 nm and using the extinction coefficient of $16,960 \text{ M}^{-1} \text{ cm}^{-1}$ for smURFP-tag. The purified protein was stored at $<200 \text{ }\mu\text{M}$ to prevent protein precipitation in sterile-filtered PBS at 4°C .

2.2. Addition of BV, BV-PEG-FAM, & BV-(PEG-FAM)₂ to smURFP-tag *in vitro*

smURFP-tag without chromophore was diluted to $1 \text{ }\mu\text{M}$ in PBS, pH 7.4 + 10% FBS. A 10-fold excess of the chromophore ($10 \text{ }\mu\text{M}$ of BV, **BV-PEG-FAM**, and **BV-(PEG-FAM)₂**) was added to the smURFP in PBS + 10% FBS from 5 mM DMSO stock solutions. The solutions were immediately vortexed and incubated overnight at 37°C with shaking to avoid precipitation of the BV substrates. The smURFP + chromophore (BV, **BV-PEG-FAM**, and **BV-(PEG-FAM)₂**) was purified on NiNTA beads as described above to remove non-covalently attached BV substrates. smURFP-tag + BV and **BV-PEG-FAM** were a blue color indicative of covalent attachment, while smURFP-tag + **BV-(PEG-FAM)₂** showed no visible color and lacked covalent attachment *in vitro*. Absorbance was measured on a NanoDrop One^c (ThermoFisher ND-ONEC-W) using $2 \text{ }\mu\text{l}$ of purified protein blanked with $2 \text{ }\mu\text{l}$ PBS (**Supporting Fig. 7**). Absorbance at 492 nm confirmed the presence of **BV-PEG-FAM**, and the **FAM** remains attached after covalent attachment to the smURFP-tag *in vitro*. smURFP-tag + **BV-(PEG-FAM)₂** did not show absorbance from 340-700 nm, and the substrate is not covalently attached *in vitro*.



Supporting Fig. 7 Absorbance spectra of smURFP-tag + BV (black) or **BV-PEG-FAM** (red). Absorbance was normalized to the BV maximum absorbance of 642 nm when attached to smURFP-tag. The **FAM** peak is clearly visible at 492 nm (red), but smaller than the BV peak at 642 nm due to the difference in extinction coefficient of $70,000$ and $180,000 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. smURFP + **BV-(PEG-FAM)₂** showed no absorbance from 340-700 nm and was not plotted on the graph.

3. Fluorescence Imaging of Cells

3.1. Transient transfection of HEK293A cells

smURFP-tag with nuclear localization signal (NLS), or smURFP-hCdtI(30/120) in the pCDNA3 plasmid under the cytomegalovirus (CMV) promoter was prepared as previously published¹. 2 μg of pCDNA3 CMV smURFP-hCdtI(30/120), referred to as smURFP-tag in the main text, was cationic lipid-mediated transfected with Lipofectamine 3000 (ThermoFisher L300000) in Opti-MEM into 80% confluent HEK293A cells grown on poly-D-lysine-coated glass-bottom culture imaging dish (MatTek P35G-0-14-C) in DMEM with no phenol red + 10% FBS + 1x Penicillin, Streptomycin + 1x GlutaMAX. Transfection solutions were not removed until chromophore addition 48 h later. Cells were grown at 37 °C, 5% CO₂, and 95% humidity.

3.2. Addition of biliverdin substrates to cells

Synthesized biliverdin substrates were dissolved in DMSO-d₆ or DMSO at 5 mM concentration with vigorous mixing for 15 min to ensure all material was dissolved. Concentration was confirmed by UV-Vis absorbance on a NanoDrop One^c (ThermoFisher ND-ONEC-W) by measuring the Soret band at 385 nm and using the extinction coefficient of 39,900 M⁻¹ cm⁻¹. Biliverdin substrates were added to 2 ml fresh DMEM with no phenol red + 10% FBS + 1x Penicillin, Streptomycin + 1x GlutaMAX warmed to 37 °C and mixed prior to addition to aspirated adherent HEK293A cells transfected with pCDNA3 CMV smURFP-hCdtI(30/120) or nuclear-localized smURFP-tag. Untransfected HEK293A cells were used as a control for the background fluorescence of BV substrates.

3.3. Fluorescence imaging of living cells and quantification

After 4 h incubation in biliverdin substrates, 2 μl of 10 mg/mL Hoechst 3342 stain was added to HEK293A cells with biliverdin substrates in 2 mL of DMEM with no phenol red + 10% FBS + 1x Penicillin, Streptomycin + 1x GlutaMAX and left for 10 min. Cells were immediately imaged without removal of the biliverdin substrates on an epifluorescence microscope. Fluorescence was imaged using a 40X, 1.2 numerical aperture (NA) C-apochromat, oil-immersion objective on a Zeiss Axiovert 200M inverted microscope using SlideBook software. The sample was excited with a 150 W xenon arc lamp with the following filter sets (excitation/emission): Hoechst 33342 stain 370(36)/475(40) nm, FAM 495(10)/535(25) nm, smURFP 628(40)/680(30) nm, and FAM FRET to smURFP 495(10)/680(30) nm. Camera exposure was 50 ms for Hoechst 3342 and 250 ms for all biliverdin substrates excluding 25 μM **BV-PEG-FAM** added to smURFP-tag (**Fig. 3**), which used 50 ms. NIH ImageJ² was used to calculate the mean fluorescence intensity for each sample with $n = 40$ cells. Mean fluorescence intensity is assumed to be linear with exposure time and calculated for full 250 ms exposure. Data were plotted in **Fig. 4** using KaleidaGraph 4.5 (Synergy Software). Samples were compared using a

one-way ANOVA with $\alpha = 0.05$ and a post hoc Tukey honestly significant difference (HSD) in KaleidaGraph 4.5. The calculated p values are written in **Fig. 4**.

Notes and references

- 1 E. A. Rodriguez, G. N. Tran, L. A. Gross, J. L. Crisp, X. Shu, J. Y. Lin and R. Y. Tsien, *Nat. Methods*, 2016, **13**, 763-769.
- 2 C. A. Schneider, W. S. Rasband and K. W. Eliceiri, *Nat. Methods*, 2012, **9**, 671-675.